Investigations on the effect of *Terminalia Chebula* and *Moringa Oleifera* on reproductive functions of Male Mice *Mus Musculus*

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By

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INTRODUCTION

Plants contribute immensely to human health care particularly in developing countries. Medicinal plants have a long history of indigenous use in India as well as in other countries (Hiremath *et al*., 2000a). Drugs of herbal origin derived from the whole plant (Rasonavivo *et al*., 1992; Joy *et al*., 2001) are used in the traditional system of medicines such as *Unani* and *Ayurveda* in India since ancient times. Numerous plants historically used to reduce fertility are tested scientifically (Revathi *et al.,* 2010; Vongtau *et al.,* 2005, Oluyemi *et al.,* 2007, Norrizah *et al.,* 2012, Akbarsha and Murugaian 2000).

In the last few years a marked decrease in the quality of semen has been reported (Carlsen *et al*., 1992). Approximately 30% of couple's infertility are due to male factors (Isidori *et al*., 2006). Male factor infertility has been estimated to account for approximately 50% of all problems with fertility (Ogunlesi *et al*., 2009). Drug treatment, chemotherapy, toxins, air pollution and insufficient vitamin intake are some of factors which have been reported to affect the process of spermatogenesis and the normal production of sperms (Mosher and Pratt, 1991). A large number of plants have been tested for the possible fertility regulatory properties and have been screened for their antifertility effects **(**Garg *et al*., 1998; Hiremath *et al.,* 1999, 2000b; Lohiya, 2000; WHO, 2000; Madhumitha and Saral, 2009; Bhatia *et al*., 2010**).**

Male reproductive toxicology has recently become a rapidly extending area of research particularly in India and there has been a growing concern about the effects of either synthetic or natural products on male reproductive health (Joshi *et al*., 2005). Natural plant substances possessing mild inherent androgenic and anti-androgenic properties offer effective non-conventional sources of contraception with less side effects (Duke, 1985; Naranjo, 1995) and a large number of chemical agents have been recorded to lead to a total spermatogenic arrest leading to irreversible sterility (El-Kashoury and Mansour, 2007). However, a search for an orally active, safe and effective plant preparation or its compound is needed for fertility regulation due to their less side effects.

Earlier literature have documented that the hydro - alcoholic extract of flower of *Achillea santolina*; tubers of *Tropaeolum tuberosum*; the aqueous extract of leaves of *Bougainvellea spectabili*; fruits of *Piper nigrum*; fresh leaves of *Oscimum gratissimum*; leaves of *Bacopa monnirai*; leaves of *Allamanda cathartica*; bark of *Hibiscus rosasinensis*; the methanol extract

of bark of *Quassia amara*; *Abrus precatorius*; the petroleum ether, benzene and ethanol extracts of seeds of *Crotolaria juncea*; the petroleum ether extract of leaves of *Mentha arvensis*; the ethanolic extract of amalakyadi churna; pods of *Cyamposis psoralioides*; leaf stalk of *Piper beetle*; hexane extract of flower buds of *Syzygium aromaticum* (Reddy *et al*., 1997; Sarkar *et al*., 2000; Sharma and Jacob., 2001; Seetharam *et al*., 2003; Golalipour *et al.*, 2004; Vijaykumar *et al.*, 2004; Faisal *et al.*, 2006; Singh and Singh., 2008; Jahan *et al*., 2009; Mishra *et al.*, 2009; Mishra and Singh., 2009 ; Singh and Singh., 2009; Obianime *et al.*, 2010 ; Thejashwini *et al*., 2012; Vasquez *et al*., 2012) possess antiandrogenic and antispermatogenic properties in male mice, *Mus musculus*.

Terminalia chebula Retz. (Family: Combretaceae) is a medicinal plant used in traditional medicine. The fruits of *T. chebula* are commonly called Haritaki. The pericarp of the dried fruit is used in the preparation of well known Ayurvedic formulations such as triphala, amalakyadi churna, Abhayamodaka (Sharma and Dash, 1998). Traditionally the dried fruits of chebulic myrobalan are used in treating asthma, sore throat, vomiting, diarrhea, piles, gout, heart and bladder diseases (Kirtikar *et al.*, 1935; Nadkarni., 1976; Parekh and Chanda., 2007; Bag *et al.*, 2009).

Moringa oleifera (Family: Moringaceae) is an another important medicinal herb. It is considered as “miracle tree” and is commonly known as drum stick tree. The leaves, fruit, flowers and immature pods of this tree are used as a highly nutritive vegetable, particularly in India (D’souza and Kulkarni, 1993; Anwar and Bhanger, 2003; Anwar *et al.*, 2005). *Moringa* leaves have been reported to be a rich source of β-carotene, protein, vitamin C, calcium and potassium and act as a good source of natural antioxidants (Dillard and German, 2000; Siddhuraju and Becker, 2003). Traditionally the plant is used as antispasmodic, stimulant, expectorant and diuretic (Mishra *et al.*, 2011).

Earlier studies on antifertility effect of an aqueous extract of fruits of *T. chebula* have documented that the long term administration of an aqueous extract of *T. chebula* leads to changes in the count, motility and morphology of the sperms, and biochemical contents of testis in rats (Krishnamurthy *et al*., 2007; Srivastav *et al.*, 2010). In addition a study on the ethanol extract of amalakyadi churna also has recorded alterations in the weight, diameter and biochemical content of the testis in Swiss albino mice (Seetharam *et al*., 2003).

However an aqueous extract of leaves (Cajuday and Poscidio, 2012) and seeds (Zade *et al*., 2013) of *M. oleifera* have been recorded to enhance sexual activity of male albino rats.

There are no reports on the effect of extracts of *T. chebula* and *M. oleifera* on the reproductive system of male mice, *Mus musculus.* The present study, therefore aimed to record the effect of the various extracts of the pericarp of the fruits of *T. chebula* and the leaves of *M. oleifera* on reproductive functions of male mice, *Mus musculus* to test in particular the antifertility effect of water and ethanol extracts of the pericarp of the fruits of the *T. chebula* and chloroform and ethanol extracts of the leaves of *M. oleifera*. The findings may be of importance to extrapolate the data for human studies since many of the physiological and biochemical pathways of the animal model studied are similar to man being a mammal.

The objectives of the present study includes

1. Screening of phytochemical constituents and to record the effect of the extract of the pericarp of the fruits of *Terminalia chebula* on reproductive functions of male mice.
2. Screening of the phytochemical constituents and to record the effect of the extracts of leaves of *Moringa oleifera* on reproductive functions of male mice.

The present work is presented in seven chapters as follows

Chapter I: Introduction and Review of Literature.

Chapter II: Materials and Methods

Chapter III: Preliminary phytochemical screening of the successive reextracts of the pericarp of the fruits of *T. chebula*.

Chapter IV: Records the effect of ethanol and water extracts of pericarp of the fruits of *T. chebula* on reproductive system of male mice.

Chapter V: Preliminary phytochemical screening of the successive re-extracts of the leaves of *M. oleifera* .

Chapter VI: Records the effect of chloroform and ethanol extracts of leaves of *M. oleifera* on reproductive system of male mice.

Chapter VII: Summary and Conclusion.

CHAPTER I: INTRODUCTION AND REVIEW OF LITERATURE

This chapter gives an introduction elaborating on the importance of plants and its application in modern and traditional medicines, and their effect on reproductive system, encompassing pharmacological and biological activities.

CHAPTER II: MATERIALS AND METHODS

2.1-Animals

Male Swiss albino mice, *Mus musculus* (Aged 2 months; 25-35 grams) were housed in polypropylene cages (47×34×20 cm) with paddy husk bedding under 12:12 hours of light/dark cycles at a temperature of 23.00 ± 2.0^{o}C under hygienic conditions maintained in the animal house of Applied Zoology Department, Mangalore University. The mice were provided with standard feed pellet (supplied by Hindustan Lever Ltd.) and water *ad libitum*.

2.2- Plant materials

The dried pericarp of the fruits of *T. chebula* Retz. were procured from local market of Mangalore Taluk South Canara, India and the leaves of *M. oleifera* Lam. was collected from the local kitchen herbal garden, Mangalore and were identified and authenticated by Professor Krishnakumar, Department of Applied Botany, Mangalore University.

2.3 -Preparation of plant extracts

The dried ripe fruits of *T. chebula* were washed, shade dried and hammered into small pieces. The outer pericarp was coarsely powdered using a blender. Likewise, the collected leaves of *M. oliefera* were washed, shade dried and finely powdered using a grinder. The samples were stored separately in an airtight container at 4^{o}C in a refrigerator for subsequent extraction.

About 100 grams each of the dried powder of pericarp of the fruits of *T. chebula* and the leaves of *M. oleifera* were subjected for soxhlet extraction separately using the petroleum ether, chloroform, absolute alcohol and water as solvents in succession until in each case the solvent became clear in the condenser.

The solvent extracts in each case were evaporated on a water bath to obtain a semisolid mass. The yield of each successive re-extracts were calculated and stored in an airtight container at 4^{o}C in the refrigerator till further use.

2.4 -Preliminary phytochemical screening

The various re-extracts of pericarp of the fruits of *T. chebula* and the leaves of *M. oleifera* were subjected to phytochemical analysis using the mother solvent in each case to record the presence of alkaloids, phytosterols, triterpenoids, tannins, cardiac glycosides, anthraquinones, saponins, flavonoids, carbohydrates and proteins following the standard methods (Harborne, 1998 and Kokate, 2005).

2.5 - Selection of the dose

A group of six mice (experimental) were fed with a graded dose of 0.25, 0.5, 1.0, 1.5, and 2.0 g/kg body weight of various extract and observed for mortality and behavioral response for 48 hrs. No mortality or a change in the behavioral response was recorded in experimental mice up to 2000 mg/kg b.w. Hence the $1/10^{th}$ of the maximum dose (200 mg/kg b.w.) and a double dose (400 mg/kg b.w.) was selected as a treatment dose.

The animals were fed with ethanol (0.1% carboxy methyl cellulose as a vehicle solvent) and aqueous (water as a vehicle solvent) extracts of pericarp of the fruits of *T. chebula* and chloroform (0.1% tween 80 as vehicle solvent) and ethanol (0.1% carboxy methyl cellulose as a vehicle solvent) extracts of leaves of *M. oleifera* at a dose of 200 and 400 mg/kg body weight per day for 35 days. The control mice were fed an equivalent volume of respective vehicle solvent.

2.6- Autopsy Schedule

The control and experimental animals were sacrificed by cervical dislocation on 36^{th} day following oral feeding of the extracts in each case.

2.6.1. Weight of total body, testis and accessory sex organs

The initial and final body weight of control and the experimental groups were recorded on alternative days. The reproductive organs of both of the right and left side of the body (testis, epididymis, vas deferens, cowper's gland), seminal vesicle and ventral prostate gland were dissected out carefully, freed from adherent tissues and blood and the weight of the organs recorded were expressed as mg/100gm body weight.

2.6.2. Sperm suspension/sperm count/sperm shape abnormality assay

The caudal epididymis (of the left and right side) excised separately were washed in 0.9% saline and minced using 0.5 ml of phosphate buffer saline (pH 7) and filtered through cheese cloth to exclude tissue debris. The filtrate (sperm suspension) obtained was used to determine the sperm count and to assess sperm abnormalities.

The sperm count was enumerated using the method of Vega *et al*. (1988). The sperm suspension was drawn to the 0.5 mark of WBC pipette and the phosphate buffer saline was drawn up to the mark 11 and mixed well. The **sperm** suspension was loaded into improved Neubauer counting chamber and was allowed to settle.

The sperm shape abnormalities were assessed following the criteria described by Wyrobek and Bruce (1975). To 10 parts of sperm suspension was added 1 part of 1% aqueous eosin and the smear of sperm suspension was prepared after 30 minutes. The slides were air dried and observed under compound microscope using 45X objective lens. A total of 2000 sperms per animal were scored. The number of abnormal sperms were expressed as percent of the total counts.

2.6.3. Biochemical analysis

The testis of the left side was used to estimate the level of acid and alkaline phosphatase (Eichholz and Crane, 1965) and protein (Lowry *et al*., 1951).Whereas the testis of the right side was used to estimate glycogen (Seifter, 1957**).** The DNA content was estimated using the modified diphenylamine method (Burton, 1956) and RNA content by using orcinol method (Ceriotti, 1955). The fructose content of the seminal vesicle was determined using the method of Mann (1964).

2.6.4 -Blood parameters: Collection of blood /serum samples

Blood samples from mice were collected via cardiac puncture into heparinised (bood) and non heparinised (serum) bottles separately. The serum collected was used to analyze cholesterol **(**Flegg, 1973) creatinine (Haeckel *et al*., 1981), serum glutamic oxaloacetic transaminase

(SGOT) and serum glutamic pyruvate transaminase (SGPT) using biochemical kits of AGAPPE Diagnostics.

The whole blood was analyzed for urea using diacetyl monoxime method (Rosenthal, 1955); hemoglobin (Drabkin and Austin, 1932); red blood corpuscles (RBC); white blood corpuscles (WBC); differential counts; packed cell volume (PCV) and erythrocyte sedimentation rate (ESR) using Automated cell counter-sysmix KX-21.

2.6.5 -Histological analysis of testis

The testis of control and treated mice were fixed in aqueous Bouin's fixative. The standard procedure was followed to prepare the paraffin blocks and the sections taken (5 µm thick) were stained using haematoxylin and eosin stain (Bacroft and Stevents, 1996). The sections were observed and analysed to record changes in the diameter of testis, seminiferous tubules and the primary and secondary spermatogonial cells. The sections were photographed using Olympus BX51 microscope (40X magnification) and Jenoptik ProgRes C3 camera.

2.6.6 - Fertility test of male mice

The treated male mouse was co-habited for ten days with three virgin untreated females of the same strain and the numbers of litters born in each case were counted.

2.13- Statistical analysis:

Data is expressed as mean ± SEM. Students t-test analysis was applied to test the significance of difference between the results of the treated and control groups. The differences were considered significant at the conventional levels of significance $P<0.001$, $P<0.01$, and $P<0.05$.

CHAPTER III: PHYTOCHEMICAL SCREENING OF SUCCESSIVE RE-EXTRACTS OF THE PERICARP OF FRUITS OF *TERMINALIA CHEBULA* RETZ.

The pericarp of the fruits of *T. chebula* was extracted using petroleum ether, chloroform, ethanol and water solvents in succession .The successive re-extracts obtained in each case were analysed to screen the presence of various phytoconstituents.

Result

The phytochemical tests employed indicate the presence of hydrolysable tannins in ethanol and aqueous extracts and the absence in the petroleum ether and chloroform extracts. The Dragendorff's, test for alkaloids was positive in all the extracts and the Hager's was highest in chloroform and negligible in ethanol and low in aqueous extracts whereas Mayer's test was negative in petroleum ether and chloroform extracts and negligible in ethanol and aqueous extracts. The Liebermann- Burchard's test for phytosterols was positive in petroleum ether and chloroform and negative in ethanol and aqueous extracts. The Salkowski test for triterpenoid was positive in all extracts exhibiting negligible amount of triterpenoids in chloroform extract. The shinoda and alkaline reagent test for flavonoids were negative in petroleum ether extract only. The shinoda test indicated the highest amount of flavonoids in chloroform whereas the alkaline reagent test exhibited the highest amount in ethanol extract. The foam and olive oil tests for saponins were positive only in aqueous extract. The hydroxyanthroquinone test was positive in chloroform, ethanol and aqueous extracts and negative in petroleum ether extract. The Molisch tests for carbohydrates indicated the presence of a very low amount of carbohydrates in petroleum ether compared to other extracts. However, Fehling's tests was negative only in petroleum ether and Barfoed's test was negative both in the case of petroleum ether and chloroform extracts exhibiting the absence of the reducing sugars. The Seliwanoff's test indicated the presence of keto sugars in all the extracts exhibiting low amount in petroleum ether extract. The Xanthoproteic (specific for nitro derivatives of the aromatic amino acids) and Sakaguchi tests (specific for guanidine group containing amino acid arginine) were positive in ethanol and aqueous extracts whereas Millon's test (hydroxyl phenol group of tyrosine) was positive only in ethanol extract. Tests for protein (Biuret), cardiac glycosides (Kellar-Killiani's) and fats and fixed oils were negative in all the successive extracts.

Conclusion

In particular the ethanol and aqueous extract shared the presence of tannins alkaloids, triterpenoids, flavonoids, hydroxyanthroquinone glycosides, carbohydrates, amino acids and the absence of phytosterols, cardiac glycosides, proteins, fixed oils and fats. Saponins, was present only in aqueous extract.

CHAPTER IV: EFFECT OF *TERMINALIA CHEBULA* EXTRACTS (AQUEOUS AND ETHANOL) ON REPRODUCTIVE SYSTEM OF MALE MICE

The aqueous extract of pericarp of the fruits of *T. chebula,* was dissolved in water and the ethanol extract was dissolved in 0.1% CMC (carboxy methyl cellulose) and were fed orally to male mice at the dose level of 200 and 400 mg/kg b.w. for 35 days. The control animals received the same volume of respective vehicle. The animals were divided into six groups as detailed below:

Experimental Design

Group I: Water control group - received 0.1 ml of distilled water orally per day for 35 days.

Group II: Experimental mice – received 200 mg/kg body weight of aqueous extract of *Terminalia chebula* suspended in 0.1 ml of distilled water orally per day for 35 days.

Group III: Experimental mice - received 400 mg/kg body weight of aqueous extract *Terminalia chebula* suspended in 0.1 ml of distilled water orally per day for 35 days.

Group IV: CMC control group -received 0.1 ml of CMC (0.1%) orally per day for 35 days.

Group V: Experimental mice - received 200 mg/kg body weight of ethanolic extract of *Terminalia chebula* suspended in 0.1 ml CMC (0.1%) orally per day for 35 days.

Group VI: Experimental mice - received 400 mg/kg body weight of ethanolic extract of *Terminalia chebula* suspended in 0.1 ml of CMC (0.1%) orally per day for 35 days.

The effect of aqueous extract of the pericarp of fruits of *T. chebula* on reproductive system was compared with Group I (water control) and the ethanol extract with Group IV (Vehicle control).

Results

Effect on body weight

The experimental mice (group II and III) administered orally the **aqueous extract** of pericarp of the fruit of *T. chebula* a dose of 200 and 400 mg /kg body weight exhibited an increase of 43 and 17% in total body weight. However 22% increase in the body weight was recorded in the control mice (group-I).

The experimental mice (group II and III) administered orally a dose of 200 and 400mg/kg body weight of **ethanol extract** of pericarp of fruit of *T. chebula* exhibited an increase of 15 and 16% in total body weight respectively.

Effect on reproductive organ weight

A significant decrease was recorded in weight of **testis** in mice fed with **aqueous extract** at a dose of 200 mg/kg body weight. However, the decrease in the weight of the left testis was more significant compared to right testis whereas the weight of both the right and left testis exhibited a nonsignificant increase in experimental mice group (III) fed a dose of 400 mg/kg body weight.

On the other hand, in experimental mice fed with **ethanol extract** of pericap of fruits of *T. chebula* exhibited a significant decrease in weight of the left testis. Whereas, the decrease was significant in both the right and the left testis in mice fed a dose of 400 mg/kg body weight. However, the decrease in the weight of the left testis was more significant compared to right testis in both the treated mice fed a dose of 200 and 400 mg/kg b.w. of ethanol extract.

The weights of the caudal and caput epididymis, vas deferens, cowper's gland and seminal vesicle did not exhibit any significant change in mice fed both with 200 and 400 mg/kg body weight of **aqueous extract.** The decrease in the weight of the prostate gland recorded was significant only in the mice administered with 400 mg/kg body weight compared to control.

The weights of caudal epididymis and vas deferens decreased significantly, whereas the weight of caput epididymis, cowper's gland, seminal vesicle and prostate gland did not exhibit any significant change in mice fed with a dose of 200 mg/kg body weight of **ethanol extract**. On the other hand, a significant decrease in the weight of caudal epididymis (right), caput epididymis (right and left) and seminal vesicle was recorded in mice fed with 400 mg/kg body weight of the extract. However, the weights of left caudal epididymis, vas deferens, cowper's gland and prostate gland did not exhibit any significant change compared to CMC control group.

Effect on Sperm count

No significant change was recorded in sperm count of cauda epididymis in mice fed a dose of 200 and 400 mg/kg b.w. of aqueous and ethanol extract compared to respective control groups.

Effect on sperm shape morphology

The total percent of sperm shape abnormalities did not exhibit any significant change in mice fed with 200 mg/kg b.w. **aqueous extract**. Whereas a significant ($p<0.001$) increase in sperm shape abnormalities were recorded in mice fed orally a dose of 400 mg/kg b.w. compared to water control group. In particular, the hookless type of sperms increased significantly ($p<0.01$) in mice fed with 200 mg/kg b.w. whereas the folded types of sperms exhibited a significant increase ($p<0.001$) in mice fed a dose of 400 mg/kg body weight of aqueous extract compared to water control group.

In experimental mice group (V) fed with 200 mg /kg b.w. of **ethanol extract** recorded a significant ($p<0.001$) increase in the total percent of sperm shape abnormalities. Whereas a increase in sperm shape abnormalities was nonsignificant in mice fed orally a dose of 400 mg/kg body weight of ethanol extract compared to CMC control group. The amorphous, banana and hookless sperm types increased significantly in mice fed a dose of 200 mg/kg of ethanol extract. Whereas, amorphous and folded tail types of sperms increased significantly in mice fed a dose of 400 mg/kg body weight of ethanol extract.

Effect on biochemical parameters of testis

The experimental mice fed with both 200 and 400mg/kg b.w. of **aqueous extract** of pericarp of the fruits of *T. chebula.* did not exhibit any significant change in the level of **Alkaline phosphatase** (**ALP), protein,** and **DNA** content of the testis. However, the activity of **acid phosphatase (ACP)**, exhibited a significant ($p<0.05$) decrease in mice fed orally a dose of 400 mg/kg b.w. of extract. A significant ($p< 0.01$) increase was recorded in the **glycogen** content of testis in mice fed with 200 mg/kg b.w. The **RNA** content of testis exhibited significant ($p<0.05$) increase in mice fed with 200 mg and a significant ($p<0.01$) decrease in mice fed with 400 mg/kg b.w. of ethanol extract. The **fructose** content of seminal vesicle decreased significantly both in

mice groups fed orally with 200 and 400 mg/kg b.w. of aqueous extract compared with water control group.

The **experimental mice** fed a dose of 200 and 400 mg/kg b.w**. of ethanol extract** exhibited no significant change in the **glycogen** content of testis. A significant decrease in the **acid and alkaline phosphatase** activity was recorded in both 200 and 400 mg/kg b.w. of extract. The **protein** content of testis exhibited a significant ($p<0.01$) increase in mice fed with 200 mg/kg b.w. and significant ($p<0.001$) decrease in 400 mg/kg b.w. of ethanol extract. The **DNA** content of testis exhibited a non significant decrease in mice fed with 200 mg/kg b.w. dose. Whereas a significant ($p<0.001$) decrease in DNA content was recorded in mice fed with 400 mg/kg of extract. The **RNA** content of testis increased significantly ($p<0.01$) in mice fed with 200mg/kg b.w. dose. Whereas a significant ($p<0.05$) decrease in RNA content was recorded in mice fed a dose of 400 mg/kg b.w. The **fructose** content of seminal vesicle exhibited a significant ($p<0.01$) decrease in mice treated with 200mg/kg b.w. Whereas a significant ($p<0.001$) increase was recorded in mice fed with 400mg/kg b.w.of ethanol extract compared with CMC control group.

Effect on serum parameters

Experimental mice fed with both 200 and 400mg/kg b.w. of **aqueous extract** of the pericarp of fruits of *T. chebula* did not exhibit any significant change in the level of serum **cholesterol, creatinine** and **SGOT**. The level of serum **SGPT** exhibited a significant decrease in mice fed with 200 mg/kg b.w. However, the decrease in SGPT level was non significant in mice fed a dose of 400 mg/kg b.w. of extract compared to vehicle control.

Experimental mice, fed with both 200 and 400mg/kg b.w. of **ethanol extract** of pericarp of the fruits of *Terminalia chebula* did not exhibit any significant change in the levels of serum **cholesterol, creatinine and SGPT,** Whereas, the level of serum SGOT exhibited a significant increase in mice administered with 400 mg/kg b.w. of extract. However the level of serum SGOT exhibited a non significant decrease in mice administered with 200mg/kg b.w. of extract.

Effect on blood parameters

The effect of **aqueous and ethanol** extracts of the pericarp of the fruits of *T. chebula* on blood parameters (Blood urea, haemoglobin, WBC, differential count, RBC, ESR, PCV) of control groups (I & IV) and experimental groups (II, III, V & VI) recorded a nonsignificant changes in the level of blood urea, neutrophil ,lymphocyte, eosinophil, ESR, PCV and RBC count of blood in mice fed with both 200 and 400 mg/kg b.w. of aqueous extract. However the total WBC count exhibited a significant ($p<0.05$) increase in mice fed with 200 mg/kg b.w. Whereas the total WBC count remain unchanged in mice fed with 400 mg dose. The blood haemoglobin level exhibited a significant ($p<0.05$) decrease in mice fed with 400mg/kg b.w. of extract and the concentration of haemoglobin remain unchanged in mice fed with 200 mg/kg b.w. of **aqueous extract.**

On the other hand, the level of blood haemoglobin, total WBC, number of neutrophil, lymphocyte, ESR, PCV and RBC count did not exhibit any significant change in mice fed with both 200 and 400mg/kg b.w. of **ethanol extract**. Whereas the level of blood urea exhibited a significant ($p<0.01$) increase and the percent of eosinophil exhibited a significant ($p< 0.05$) decrease in mice fed with 400 mg/kg body weight of extract. However non significant change was exhibited in the level of blood urea and eosinophil in mice fed with 200mg/kg b.w. of **ethanol extract** compared to CMC control group.

Effect on histological parameters of testis

The effect of **aqueous extracts** of the pericarp of the fruits of *T. chebula* on histology of testis of control and treated mice exhibited a significant ($p<0.001$) decrease in the overall diameter of testis in mice administered with 200 mg/kg body weight of aqueous extract. Whereas, the decrease in diameter of testis was non significant in mice administrated with 400mg/kg b.w. The diameter of the seminiferous tubule and primary spermatogonial cells exhibited a significant decrease in both mice groups fed with 200 and 400 mg/kg b.w.of aqueous extract. However, the secondary spermatogonial diameter did not exhibit any significant changes in both the doses.

A significant decrease in the diameter of testis was recorded in mice fed with both 200 and 400mg/kg b.w. of **ethanol extract.** The result exhibited a nonsignificant changes in the diameter of seminiferous tubule, primary and secondary spermatogonial cells compared to CMC control mice.

Effect on Fertility rate of male mice

A significant ($p<0.05$) reduction in the number of litters was recorded in mice administered orally with 200 mg (group II) and 400 mg/kg b.w. (group III) of **aqueous extract** of pericarp of the fruits of *T. chebula.*

The decrease in the number of litters born was significant ($p<0.05$) in mice administered orally with 200 mg/kg (group V) of **ethanol extract**. However, the reduction in number of litters in mice fed with 400 mg/kg b.w. (group VI) of ethanol extract was non significant compared to CMC control group (IV).

Conclusion

The overall result indicates that, the water and ethanol extracts of pericarp of the fruits of *T. chebula* caused significant impairment in the spermatogenesis and spermeogenesis process of mice. This may be attributed to the presence of potent antifertility agents in the plant extracts.

CHAPTER V: PHYTOCHEMICAL SCREENING OF SUCCESSIVE EXTRACTS OF LEAVES OF *MORINGA OLEIFERA*.

The leaves of *M. oleifera* was extracted using petroleum ether, chloroform, ethanol and water solvents in succession. The successive extracts collected in each case were subjected to screen the presence of various phytoconstituents.

Result

The phytochemical tests employed indicated the presence of hydrolysable tannins in ethanol and aqueous extracts and their absence in petroleum ether and chloroform extracts. The Dragendorff's test for alkaloids was positive in ethanol and aqueous extracts whereas the Hager's, Mayer's and the Wagner tests exhibited the presence of low amount of alkaloids only in the ethanol extract. The Libermann-Burchard's test for phytosterols was positive in petroleum ether and chloroform extracts and negative in ethanol and aqueous extracts. The Salkowski test

for triterpenoid was positive only in ethanol and aqueous extracts. The lead acetate and alkaline reagent tests indicated the presence of high amount of flavonoids in ethanol and water extracts whereas the Shinoda test exhibited the presence of low amount of flavonoids in aqueous extracts. The Foam test was negative whereas the olive oil test for saponin was positive in aqueous extract. The Kellar killani test for cardiac glycoside was positive in petroleum ether, chloroform and ethanol extracts and negative in aqueous extracts. Hydroxy-anthroquinone test was positive in all the extracts. The Molisch's and Fehling's test indicated the presence of high amount of carbohydrates in the ethanol and aqueous extracts whereas the Barfoed's test exhibited low amount of carbohydrates in ethanol extract and negative in aqueous extract. The Seliwanoff's test indicated the presence of keto sugars in all extracts exhibiting lowest amount in chloroform extract. Millon's test for amino acid (hydroxyl phenol group of tyrosine) was positive in ethanol and aqueous extracts.The tests for protein (Biuret) and fats and fixed oils were negative in all the successive extracts. The results suggest the presence of tannins, alkaloids steroids, triterpenoids, flavonoids, hydroxy-anthraquinones, cardiac glycosides, saponins, and carbohydrates (glucose and fructose) having selective solubility in selected solvents of varying polarities used in succession. However, the tests for proteins and fixed oils and fats were negative in all the extracts.

Conclusion

In particular the chloroform and ethanol extract shared the presence of cardiac glycoside and hydroxyanthroquinone, and the absence of saponins and proteins. Whereas, tannins, alkaloids, triterpenoids, flavonoides, amino acids present in ethanol extract and phytosterols were present only in the chloroform extract.

CHAPTER VI: EFFECT OF *MORINGA OLEIFERA* LEAF EXTRACTS (CHLOROFORM AND ETHANOL) ON REPRODUCTIVE SYSTEM OF MALE MICE.

The chloroform extract of the of the leaves of *Moringa oleifera* dissolved in 0.1% Tween 80 whereas the ethanol extract was dissolved in 0.1% carboxy methyl cellulose were fed orally to male mice at a dose of 200 and 400 mg/kg body weight per day for 35 days. The control animals received the same volume of respective vehicle solvent. The control and the treated mice were categorized into six groups as detailed below:

Experimental design

Group I: Water control group received 0.1 ml of distilled water orally per day for 35 days.

Group II: Tween 80 control group received 0.1 ml of 0.1% Tween 80 orally per day for 35 days.
Group III: Experimental mice received 200mg/kg body weight of chloroform extract of *M. oleifera* suspended in 0.1 ml of 0.1% Tween 80 orally per day for 35 days.
Group IV: Experimental mice received 400 mg/kg body weight of chloroform extract of *M. oleifera* suspended in 0.1 ml of 0.1%Tween 80 orally per day for 35 days.
Group V: CMC control group received 0.1 ml of 0.1% CMC orally per day for 35 days.
Group VI: Experimental mice received 200 mg /kg body weight of ethanol extract of *M. oleifera* suspended in 0.1 ml 0.1% CMC orally per day for 35 days.
Group VII: Experimental mice received 400 mg/kg body weight of ethanol extract of *M. oleifera* suspended in 0.1 ml 0.1% CMC orally per day for 35 days.

Results

Effect on body weight

The decrease in body weight of experimental mice (Group III and IV) fed orally a dose of 200 and 400mg/kg body weight of **chloroform extract** of the leaves of *M. oleifera* recorded an overall decrease of 5 and 4% respectively. However, an increase of 13.5% was recorded in the body weight of control (Group II) mice compared to initial body weight.

The experimental mice fed a dose of 200 (Group II) and 400mg/kg b.w. (Group III) of chloroform extract of the leaves of *M. oleifera* however recorded a decrease of approximately 60 and 70% in body weight respectively compared to vehicle control mice.

On the other hand, the respective control (group V) and experimental (group VI and VII) mice fed a dose of 200 and 400 mg/kg b.w. of **ethanol extract** of the leaves of *M. oleifera* recorded an increase of 20, 22, 15% compared to initial body weight.

The body weight of the experimental mice fed a dose of 200 and 400 mg/kg body weight of ethanol extract compared to control group (V) recorded a non significant increase of 8% and a non significant decrease of 25% in body weight respectively compared to CMC control group.

It points that the chloroform and ethanol extract of *M. oleifera* leaves are non toxic at both the doses. In the present study however the animals in the control group gained more weight compared to experimental group and the weight gained decreased with graded dose in ethanol extract. Whereas the weight gain is less in experimental groups fed a higher dose of 400 mg/kg body weight of chloroform extract compared to the lower dose of 200 mg / kg body weight of the extract.

Effect on reproductive organ weight

A significant decrease was recorded in the weight of the testis in experimental mice fed a dose of 200 and 400 mg/kg body weight of **chloroform e**xtract of the leaves of *M. oleifera*. The decrease however was more significant in left testis in mice fed a dose of 200 mg and in right testis in mice fed a dose of 400 mg/kg body weight.

On the other hand, in experimental mice fed a dose of 200 mg/kg b.w. of **ethanol extract** of the leaves of *M. oleifera* exhibited a significant ($p< 0.01$) decrease in the weight of the left testis.

In experimental mice (group III and IV) fed a dose of both 200 and 400 mg/kg b.w. of the **chloroform extract** exhibited a significant decrease in the weight of right caudal epididymis, caput epididymis, vas deferens, cowper's gland, prostate gland and seminal vesicle. Whereas the decrease recorded in the weight of left caudal epididymis was nonsignificant in the mice fed with 200 mg /kg body weight of extract compared to control group.

In experimental mice (group III and IV) fed a dose of both 200 and 400 mg/kg b.w of **ethanol extract** of leaves of *M. oleifera* exhibited a significant decrease in the weight of caudal epididymis, right vas deferens, right cowper's gland and seminal vesicle. The increase in the weight of caput epididymis was significant fed a dose of 200mg/hg b.w. Whereas the weight of left vas deferens, left cowper's gland and prostate gland did not exhibit any significant change in the mice fed a dose of 200 mg/kg b.w. However, non significant change was recorded in the weight of caput epididymis and prostate gland in experimental mice fed a dose of 400 mg/kg b.w. of ethanol extract compared to control group.

Effect on caudal epididymal sperm count

The cauda epididymal sperm count (million/ml) in experimental (Group III, IV, VI & VII) mice fed a dose of both 200 and 400mg/kg b.w. of **chloroform and ethanol extracts** of the leaves of *M. oleifera* exhibited a significant decrease compared to control group.

Effect on sperm shape morphology

The abnormal sperm types recorded in present study includes amorphous, folded tails, hookless, banana headed and double headed sperms.

The total percent of sperm shape abnormalities recorded in experimental mice fed a dose of 200 and 400 mg/kg. b.w of **chloroform extract** exhibited a significant increase compared to vehicle control. The amorphous, folded tail, hookless and double headed types of sperm abnormalities exhibited a significant increase in mice fed a dose of 200 mg/kg b.w. whereas banana and hookless types of sperms increased significantly in mice fed a dose of 400 mg/kg b.w of chloroform extract compared to respective vehicle control.

In experimental mice fed with **ethanol extract** of the leaves of the *M. oleifera* recorded a significant increase in total percent of sperm shape abnormalities in mice fed a dose of 400 mg/kg b.w. compared to vehicle control. However, the increase in sperm shape abnormalities was nonsignificant in mice fed a dose of 200 mg/kg. b.w of **ethanol extract.** The increase in folded, banana and hookless types of sperm abnormalities was significant in mice fed a dose of 200 mg/kg b.w. of ethanol extract. Whereas, the amorphous and folded tail types of sperms increased significantly in mice fed orally a dose of 400mg/kg b.w of the ethanol extract compared to vehicle control.

Effect on biochemical parameters of testis

The experimental mice fed orally with 200 mg/kg. b.w of **chloroform** extract exhibited a significant decrease in the level of **acid phosphatase (ACP)** of testis. Whereas a significant increase was recorded in the level of ACP of testis in experimental mice fed orally a dose of 400mg/kg.b.w. of extract. The level of **alkaline phosphatase (ALP)** of testis exhibited a significant decrease in experimental mice fed orally with 200 mg/kg. b.w of **chloroform** extract.

However, the decrease in the level of **alkaline phosphatase was nonsignificant** in mice fed orally a dose of 400 mg/kg.b.w. of extract. The protein content of testis exhibited a significant decrease in mice fed with 400 mg/kg b.w. However the glycogen and RNA content of testis and the fructose content of seminal vesicle exhibited a significant decrease in mice fed a dose of 400 mg/kg b.w. of extract. The DNA content of testis exhibited a significant increase in mice fed a dose of 200 mg/kg b.w of chloroform extract of leaves of *M. oleifera* compared to control.

In experimental mice administered with both 200 and 400 mg/kg b. W. of **ethanol extract** exhibited a significant increase in the level of **acid phosphatase.** However, the level of **alkaline phosphatase e**xhibited a significant increase in mice fed a dose of 200 mg, while a significant decrease was recorded in mice administered with 400 mg/kg b.w. of extract. The protein and glycogen content of testis and fructose content of seminal vesicle exhibited a significant decrease in mice administered with 200 and 400mg/kg b.w. of extract. The DNA content of testis recorded a significant increase in mice administered with 200 mg, whereas a significant decrease was recorded in DNA content in mice fed of 400 mg/kg b.w. of extract. The RNA content of testis exhibited a significant increase in mice fed a dose of 200 mg/kg b.w. of extract. However, the change in RNA content of testis in mice fed a dose of 400 mg/kg b.w. of ethanol extract was non significant compared to control group.

Effect on Serum parameters

The levels of serum cholesterol, creatinine and SGOT did not exhibit any significant change in mice fed with both 200 and 400 mg/kg b.w. of **chloroform extract** of leaves of *M. oleifera.* The serum SGPT level exhibited a significant increase in mice fed with 200 mg/kg b.w. However, the increase in SGPT level was nonsignificant in mice fed with 400 mg/kg b.w. of extract compared to vehicle control.

On the other hand the level of serum creatinine, SGOT and SGPT did not exhibit any significant change in mice administered with both 200 and 400 mg /kg bw of **ethanol extract**. However, the level of serum cholesterol exhibited a significant increase in mice fed with 200 mg/kg b.w. of extract. Whereas a non significant increase was recorded in the level of serum cholesterol in mice fed with 400mg/kg b.w. of extract compared to control mice.

Effect on blood parameters

The effect of **chloroform and ethanol** extracts of leaves of *M. oleifera* on blood parameters (blood urea, haemoglobin, total WBC, differential count, RBC, ESR, PCV level) of control (group II & V) and the experimental (Group III, IV, VI & VII) mice fed a dose of 200 and 400mg/kg b.w. of extract recorded a non significant change in the level of blood urea, haemoglobin, total WBC, lymphocyte, RBC, ESR and PCV of blood. However, the percent of neutrophil count exhibited a significant decrease in mice administered with 200 mg/kg b.w. of **chloroform extract** of leaves of *M. oleifera* compared to control group (II).

On the other hand, the levels of blood haemoglobin, urea, total WBC, differential count, ESR, PCV and RBC did not exhibit any significant change in mice fed a dose of 200 and 400 mg/kg b.w. of **ethanol extract** of leaves of *M. oleifera* compared to control group(V).

Effect on histological parameters of testis

The effect of **chloroform extract** of leaves of *M. oleifera* on histological parameters of testis of control (group II) and the experimental (Group III, IV) mice exhibited a significant decrease in the diameter of testis and seminiferous tubule in mice administered with 200 and 400 mg/kg b.w. Whereas the diameter of primary and secondary spermatogonial cells exhibited a significant decrease in mice administered with 400 mg/kg b.w. of extract. However, the decrease in diameter of spermatogonial cell was nonsignificant in mice administered with 200 mg/kg b.w of extract.

On the other hand in experimental mice fed with **ethanol extract** at a dose of 200 and 400mg/kg b.w. recorded a significant decrease in the diameter of testis, seminiferous tubule, primary and secondary spermatogonial cells compared to vehicle control mice.

Effect on Fertility rate of male mice

The effect of **chloroform and ethanol** extracts of leaves of *M. oleifera* on fertility rate in the case of experimental (Group III, IV, VI &VII) mice co-habited with females recorded a significant reductions in the number of litters born compared to control (groups II & V).

Conclusion

The present study suggests that the experimental animal treated with chloroform and ethanol extract of the leaves of the *M. oleifera* exhibited a reduction in the weights of reproductive and accessory organs, alteration in the level of biochemical variables and histological features of testis. Fertility test exhibited a reduction in the number of pups born in the case of experimental mice cohabited with females suggests that the extracts of this plants possess anti-spermatogenic properties suggesting that chloroform and ethanol extracts of the leaves of *M. oleifera* possess a potential to function as an antiferitlity agent.

CHAPTER VII: SUMMARY AND CONCLUSION

Terminalia chebula

The preliminary phytochemical screening of the ethanol and the water extract of pericarp of the fruits of *Terminalia chebula* have demonstrated the presence of tannins, alkaloids, triterpenoids, flavonoids, hydroxyanthraquinones, carbohydrates and amino acids. The water extract however, in addition have exhibited the presence of saponins also.

The mice administered orally with the water extract of the pericarp of the fruits of *T. chebula* at a dose of 200 and 400 mg/kg.b.w exhibited a decrease in the weight of the testis, prostate gland and epididymal sperm count. The reduction in the level of ACP and RNA content of the testis, fructose content of seminal vesicle, serum SGPT, blood haemoglobin. Whereas the sperm shape abnormalities and number of total WBC count increased. The histological observations recorded a decrease in the diameters of testis, seminiferous tubules and primary spermatogonia. The fertility test recorded reduction in the number of litters compared to the control group.

The mice fed orally with the ethanol extracts of the pericarp of the fruits of *T. chebula* at a dose of 200 and 400 mg/kg.b.w. have recorded an increase in the total body weight and a decrease in the weight of the reproductive organs (testis, cauda and caput epididymis, vas deferens, prostate gland and seminal vesicle), epididymal sperm count and increase in sperm shape abnormalities. The level of various biochemical parameters like alkaline phosphatase, protein, DNA and RNA content of testis exhibited significant reduction. The glycogen content of

testis exhibited a nonsignificant change. The fructose content of seminal vesicle and the eosinophil count exhibited significant reduction. Whereas an increase has been recorded in the level of blood urea and the level of serum SGOT. Histological observations have exhibited a decrease in the overall diameter of the testis. The fertility test recorded reduction in the number of litters compared to the control group.

The overall data exhibits that the ethanol and the aqueous extracts of the pericarp of the fruits of *T. chebula* administered orally at dose of 200 and 400 mg/kg.b.w. per day for 35 days, induces an impairment in the reproductive functions of male mice suggesting that the pericarp of fruit of *T. chebula* possesses antifertility properties which may be attributed to the action of certain bioactive constituents recorded in the extract of the pericarp of the fruits of *Terminalia chebula*.

Moringa oleifera

The preliminary phytochemical screening of leaves of *Moringa oleifera* have recorded the presence of tannins, alkaloids, triterpenoids, flavonoids, carbohydrates and amino acids in ethanol extract.Whereas the phytosterols were present only in chloroform extract. However the Cardiac glycosides and hydroxyanthraquinones were recorded both in chloroform and ethanol extracts.

The mice fed orally with the chloroform extract of the leaves of *M. oleifera* at a dose of 200 and 400mg/kg.b.w have recorded a decrease in the total body weight and the reductions in the weights of reproductive organs (testis, cauda and caput epididymis, vas deferens, cowper's gland, prostate gland and seminal vesicle) epididymal sperm count, reduction in the level of various biochemical parameters (alkaline phosphatase, protein ,DNA and RNA) content of the testis and fructose content of seminal vesicle, neutrophil count of blood. The percentage of sperm shape abnormalities increased. Whereas an increase in the level of acid phosphatase and glycogen content of testis was recorded. In addition the histological observations recorded a decrease in the overall diameter of the testis, in particularly the diameters of seminiferous tubules, primary and secondary spermatogonial cells. The decrease in the number of litters was

recorded in the case of mice fed both with 400mg/kg b.w. and 200mg/kg b.w. of chloroform extract.

The mice administered orally with the ethanol extracts of leaves of *M. oleifera* at a dose of 200 and 400 mg/kg.b.w. exhibited an increase in the total bodyweight, reductions in the weights of reproductive organs (testis, cauda and caput epididymis, vas deferens, cowper's gland and seminal vesicle), epididymal sperm count, level of various biochemical parameters (alkaline phosphatase, protein, glycogen, DNA content) of testis and fructose content of seminal vesicle and an increase in the percentage of sperm shape abnormalities. Whereas an increase in the level of acid phosphatase of testis and serum cholesterol was recorded. The histological parameters such as diameters of testis, seminiferous tubule, primary and secondary spermatogonia were significantly decreased compared to control. The decrease in the number of litters was recorded in the case of mice fed both with 200mg and 400mg/kg b.w. of ethanol extract.

The overall data suggests that the chloroform and ethanol extracts of the leaves of *Moringa oleifera* administered orally at dose of 200 and 400mg/kg.b.w. for the duration of 35 days, induces an impairment in the reproductive functions of male mice suggesting that the leaves of *Moringa oleifera* possess antifertility properties which may be attributed to the action of certain bioactive constituents recorded in the extract of the leaves of *Moringa oleifera* in present study.

Finally it is concluded that, an oral administration of the successive re-extracts of the aqueous and ethanol extracts of the pericarp of fruits of *T. chebula* and the chloroform and ethanol extracts of the leaves of *M. oleifera* induces antifertility effects in male mice, *Mus musculus*. The antifertility property of the plant extracts may be due to the presence of certain phytoconstituents present in the extracts and or the synergistic actions of these bioactive principles.

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